Intraepidermal Nerve Fibers Are Indicators of Small-Fiber Neuropathy in Both Diabetic and Nondiabetic Patients

GARY L. PITTENGER, PHD[1,2]
MADHUMITA RAY, MS[1]
NICULINA I. BURCUS, MS[1]
PATRICIA MCNULTY, MD[1]
BAHER BASTA, MD[1]
AARON I. VINIK, MD, PHD[1,2]

OBJECTIVE — Small-fiber neuropathies may be symptomatic yet escape detection by standard tests. We hypothesized that morphologic changes in intraepidermal nerves would correlate with clinical measures of small-fiber neuropathy.

RESEARCH DESIGN AND METHODS — We studied 25 diabetic and 23 nondiabetic patients with neuropathy defined by signs, symptoms, and quantitative testing and 20 control subjects. Skin biopsies were obtained from forearm, thigh, proximal leg, and distal leg, and nerves identified using immunofluorescence with antibody to protein gene product (PGP) 9.5.

RESULTS — Mean dendritic length (MDL) ($P < 0.01$) and intraepidermal nerve fiber density (IENF) ($P < 0.001$) progressively decreased from proximal to distal sites only in patients with neuropathy. There was a significant reduction in IENF when comparing control subjects and patient groups in the distal leg ($P < 0.001$). MDL was significantly decreased in the thigh ($P < 0.005$) and in the proximal ($P < 0.01$) and distal ($P < 0.002$) leg in patients compared with control subjects. IENF was not significantly altered in diabetic patients of <5 years' duration, but significantly decreased in patients with >5 years' duration. MDL showed a linear decrease with increasing duration of diabetes. Distal leg IENF showed significant negative correlations with warm ($P < 0.02$) and cold ($P < 0.05$) thermal threshold, heat pain ($P < 0.05$), pressure sense ($P < 0.05$), and neurological disability score total sensory ($P < 0.03$) and total neuropathy ($P < 0.03$) values.

CONCLUSIONS — IENF was not significantly altered in these patients at <5 years' duration of diabetes, but fell significantly after 5 years of diabetes. MDL exhibited a linear loss with time, suggesting a different mechanism of change. MDL and IENF together may prove a useful end point in therapeutic trials for neuropathy.

Diabetes Care 27:1974–1979, 2004

Neuropathy is a common complication of both type 1 and type 2 diabetes, with predominantly small fiber involvement beginning at the distal extremities and progressively becoming more proximal with time and duration of diabetes. "Burning" or "prickly" feet are common descriptions from diabetic neuropathy patients (1). It has recently been found (2) that ~56% of idiopathic neuropathy patients presenting with burning feet have impaired glucose tolerance, determined from no increase in fasting glucose but with significant elevation of 2-h postprandial glucose. This presentation may be related to an underlying small-fiber neuropathy. There are a number of other peripheral neuropathies with small fiber involvement that present with very similar symptoms to diabetic neuropathy. Small-fiber neuropathy is easily missed by standard electrophysiological tests, and the patient may exhibit normal strength, normal reflexes, and normal electrophysiology. We have demonstrated (3) that even in known cases of sensory neuropathy the sensitivity of quantitative sensory testing is only 88%. Therefore, standard clinical sensory examination may not be sensitive enough to detect the initial small changes, leaving the developing sensory neuropathy undiagnosed by currently standard methods. We have previously shown (3) that functional abnormalities in C-fiber physiology (e.g., decreased skin blood flow) may even precede the development of type 2 diabetes. A recent study (4) using skin biopsy also suggested that developing neuropathy might be a feature of the period of impaired glucose tolerance. Other investigators (2) have shown a decrease in intraepidermal nerve fiber density (IENF) with impaired glucose tolerance, although the groups studied were small and the overall frequency ~80%. Furthermore, there was no correlation between nerve fiber density and nerve conduction studies. Therefore, it was difficult to establish those developing neuropathy, although it was feasible that alterations in C-fiber density measurable by skin biopsy

From the [1]Department of Internal Medicine, The Strelitz Diabetes Institutes, Norfolk, Virginia; and the [2]Department of Pathology, Anatomy, and Neurobiology, Eastern Virginia Medical School, Norfolk, Virginia.

Address correspondence and reprint requests to Gary L. Pittenger, PhD, The Strelitz Diabetes Institutes, 855 W. Brambleton Ave., Norfolk, VA 23510. E-mail: pittengl@evms.edu.

Received for publication 12 September 2003 and accepted in revised form 15 May 2004.

A.V. has received consultant's fees from or served on the speaker's bureaus for Pfizer, Genentech, Merck, Eli Lilly, Athena, Bristol Meyers Squibb, Knoll Pharmaceuticals, GlaxoSmithKline Beecham, Boston Medical Technologies, Neurometrix, Guilford Pharmaceuticals, R.W. Johnson Pharmaceutical Research Institute, Takeda, TEVA Pharmaceutical Industries, and AstraZeneca; and has received grant support from GMP-Endotherapeutics, the American Diabetes Association, the U.S. Department of Housing and Urban Development, the National Aeronautics and Space Administration, Eli Lilly, Parke-Davis, AstaMedica, GlaxoSmithKline Beecham, the National Institute on Aging, and R.W. Johnson.

Abbreviations: IENF, intraepidermal nerve fiber density; MDL, mean dendritic length; PGP, protein gene product.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Clinical characteristics of the subjects by group for these studies, including clinical measures of neuropathy (modified from the methods of Dyck [15])*

	Control	Type 1 diabetes	Type 2 diabetes	No diabetes	*P*
n	20	3	19	23	—
Age (years)	43.3 ± 2.8	47.3 ± 8.6	57.0 ± 1.9*	58.9 ± 3.0*	<0.001
Weight (lb)	—	158.3 ± 38.2	186.4 ± 8.7	184.7 ± 8.2	—
HbA_{1c} (%)	<6.05	7.8 ± 1.3*	6.9 ± 0.3*	5.9 ± 0.1	<0.03
Total neuropathy score (>20 = severe)	<2	19.3 ± 1.3	38.9 ± 8.9	29.9 ± 3.7	—
Neurological disability score					
Total motor score (total = 72)	<2	2.0 ± 1.2	8.6 ± 4.2	3.7 ± 1.7	—
Total sensory score (total = 72)	<2	4.0 ± 4.0	20.3 ± 4.5	16.1 ± 1.8	—
Vibration threshold (arbitrary units)	(Normal, 0–6)	7.3 ± 6.6	8.7 ± 2.3	9.9 ± 2.1	—
Cold sense (°C)	3.0 ± 1.0	8.5 ± 2.3	4.3 ± 1.1	6.8 ± 1.0*	<0.03
Warm sense (°C)	7.8 ± 1.0	13.4 ± 2.2	10.7 ± 1.1	9.8 ± 0.9	—
Cold pain (°C)	15.7 ± 1.9	18.7 ± 4.4	19.2 ± 2.1	16.5 ± 1.9	—
Heat pain (°C)	11.9 ± 0.7	16.1 ± 1.7	14.0 ± 0.8	14.7 ± 0.7*	<0.03
Pressure sense (g)	3.9 ± 0.2	4.4 ± 0.5	4.4 ± 0.1	4.3 ± 0.2	—

Data are means ± 27 SE. *$P < 0.05$, Tukey-Kramer post hoc analysis.

would be present in clinically significant neuropathy.

Protein gene product (PGP) 9.5 is a ubiquitin hydrolase that can serve as a pan-axonal and pan-neuroendocrine marker (5). Since the description of its utility as a marker for epidermal nerve fibers in human skin (6), it has been used for identification of small nerve fibers in the dermis and epidermis in a range of small-fiber neuropathies, including HIV and idiopathic small-fiber and postherpetic neuropathies, among others (7–9). A major advantage of skin biopsy followed by visualization of nerve fibers using PGP 9.5 immunostaining is the capacity for quantitation of epidermal innervation by small nerve fibers (10–14). There are little published data on the density of small IENFs in skin of diabetic patients examining the proximal-to-distal gradient of IENF in diabetic neuropathy patients or correlating IENF with clinical measures of small- and large-fiber dysfunction. Therefore, we proposed that PGP 9.5 immunostaining of skin biopsies from multiple sites on diabetic neuropathy patients might shed some light on the time dependence and distal-to-proximal gradient development of the complication, as well as provide measures to identify patients who are losing peripheral nerve endings at an early stage, when intervention might prove more beneficial.

RESEARCH DESIGN AND METHODS

— Skin-punch biopsy and quantitative sensory testing were performed on 48 patients (3 type 1 and 22 type 2 diabetic and 23 nondiabetic) who presented at The Strelitz Diabetes Institutes with positive symptoms of neuropathy (Table 1). Pathologies of nondiabetic neuropathy patients included eight autoimmune (e.g., chronic inflammatory demyelinating polyneuropathy); two glucose intolerance; one each of ethanol induced, paraneoplastic, mononeuritis multiplex, porphyria, arsenic poisoning, and systemic lupus erythematosus; and seven idiopathic. In addition, biopsies were performed on 20 healthy control subjects for similar evaluation. None of the patients had symptomatic nephropathy or evidence of other autoimmune diseases. These studies were performed with the approval of the Eastern Virginia Medical School institutional review board, and all subjects gave informed consent.

Quantitative neuropathy testing

All patients had a complete history and physical examination, with special attention given to neurological evaluation. Neurological symptom scores and neurological disability scores were generated by completion of a modified questionnaire based on those developed by Dyck (15). Neuropathy was established by the criteria suggested by the American Academy of Neurology and the American Diabetes Association (16), including two of the following characteristics: presence of symptoms or signs, abnormal quantitative electrophysiology, abnormal electromyography, and autonomic dysfunction. Sensory testing included measures of vibration, temperature, and current perception (touch, prickling, and pain) thresholds performed as previously described (17). Autonomic function was assessed by three tests: the heart rate variability during deep breathing at six breaths per minute (E:I ratio) and the R:R variation in response to the Valsalva maneuver and postural change.

Skin biopsy

Skin-punch biopsy (3 mm) was performed under local anesthesia. Biopsies were collected from each patient from dorsal forearm, lateral thigh (10 cm proximal to patella), lateral proximal leg (10 cm distal to fibular head), and lateral distal leg (10 cm proximal to lateral malleolus). Tissue samples were immediately fixed in 2% buffered paraformaldehyde/lysine/periodate solution for 12–24 h at 4°C. They were subsequently cryoprotected in phosphate buffer with 20% glycerol overnight and frozen for later cryosectioning.

Immunofluorescence

The procedures for identifying neurons in skin biopsies used immunofluorescence techniques in a modification of the protocol described by McCarthy et al. (11). Biopsy specimens were cut into 50-μm sections on a Reichert cryostat. Melanin was bleached with a 0.25% $KMnO_4$ solution followed by a 5% oxalic acid solution. The tissues were permeabilized and blocked with a solution of Tris buffer with

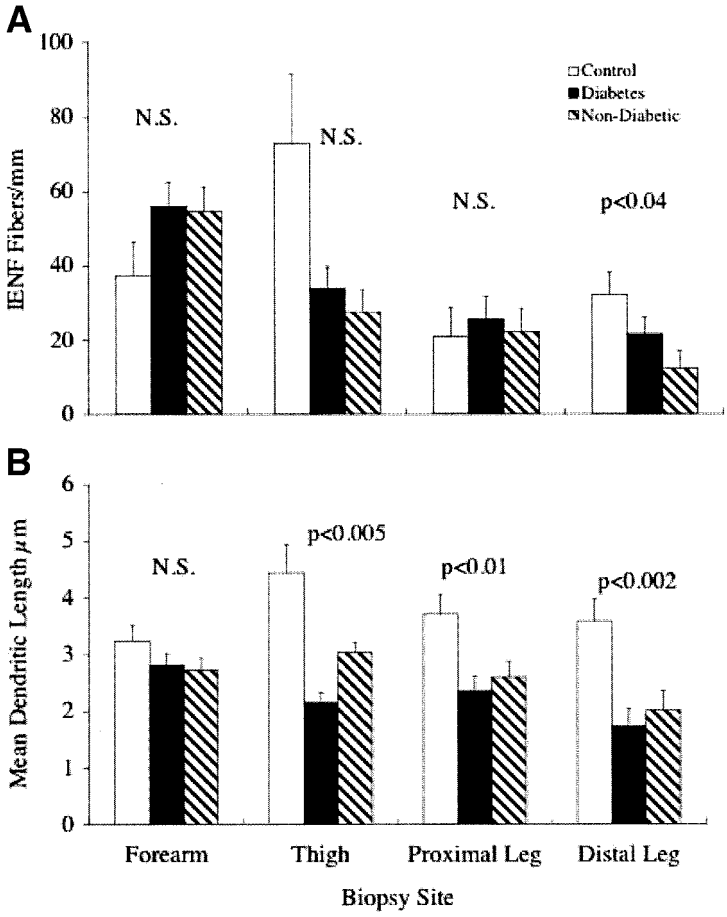

Figure 1—A: *The mean IENF density per millimeter of epidermis is significantly lower in skin from the distal leg in diabetic (■) and nondiabetic (▨) neuropathy patients compared with control subjects (□) (P < 0.05, ANOVA), but not in the forearm, thigh, or proximal leg. However, there is a progressive reduction from proximal to distal sites.* B: *The MDL is significantly lower in skin from the distal leg in diabetic (■) and nondiabetic (▨) neuropathy patients compared with control subjects (□) (P < 0.002, ANOVA). In contrast to IENF, there is also a significant difference in MDL among the groups in the thigh (P < 0.002) and proximal leg (P < 0.01).*

0.5% powdered milk, 1.0% Triton X-100, and 4% normal goat serum. Sections were stained in the same solution with rabbit anti–PGP 9.5 antibody (Chemicon, Temecula, CA) overnight at 4°C and incubated with fluorescein isothiocyanate–labeled goat anti-rabbit IgG secondary antibody for 2 h. After washing, coverslips were mounted and sealed with nail polish.

Viewing and analysis

The prepared slides were viewed and images captured by a blinded observer using an Olympus Fluoview Scanning Laser Confocal Microscope. Six random fields from at least three different sections from each specimen were collected for analysis. Confocal images were captured at 1.6-μm intervals, and the image stack was superimposed on each other to produce the image for quantitation. Quantification was performed only on the area of the photomicrographs showing epidermis. Images were analyzed for mean dendritic length (MDL) (in micrometers), mean dendrite number, and IENFs per millimeter of epidermis using Image Pro image analysis software. Results were expressed as means ± SE, and statistics were evaluated using ANOVA, with $P < 0.05$ considered significant. Post hoc Tukey-Kramer analysis was performed to compare individual groups.

RESULTS — Neuropathy symptom and disability scores as seen in Table 1 indicate that this patient population had neuropathy that was moderate to severe and predominantly sensory (15,16). There were no significant differences in neurological symptom and disability scores between diabetic and nondiabetic neuropathy groups, either as total scores or as the subscales of motor, sensory, and autonomic scores.

Quantitative sensory testing (warm and cold thermal perception threshold, heat and cold pain, and pressure sense) was performed at all four sites that were biopsied. There was a greater loss of sensory fiber function in the distal leg compared to the forearm. Cold ($P < 0.03$) and warm ($P < 0.05$) perception thresholds, as well as heat pain perception ($P < 0.02$), were significantly elevated in neuropathy patients at the distal leg compared with the control subjects, but there was no significant difference for any of the quantitative sensory measures among the groups at the forearm. This indicates that the small-fiber function was more impaired at the distal leg than the forearm in these groups of patients.

There was a significant correlation between age and distal leg MDL ($r = -0.39$, $P < 0.005$) and age and distal leg IENF ($r = -0.39$, $P < 0.005$). Data from other sites showed no relationship with age. There was a clear negative IENF gradient from proximal to distal sites in patients with neuropathy (ANOVA, $P < 0.001$) (Fig. 1*A*). In contrast, biopsies from control subjects showed only a small, nonsignificant decrease (Fig. 1*A*), even at the distal leg, where the density was lowest in neuropathy patients. Comparing results at each site, IENF in the forearm was not significantly different between patients (55.5 ± 4.5 fibers/mm tissue) and control subjects (37.4 ± 9.0, $P > 0.05$), neither was there a difference in the thigh or proximal leg. Only in the distal leg was there a significant decrease in IENF ($P < 0.05$) of patients (17.5 ± 3.3) compared with control subjects (32.3 ± 5.9).

MDL was similar across all sites for control subjects. MDL was significantly decreased in skin from neuropathy patients with a progressive decrease from proximal to distal sites ($P < 0.002$) (Fig. 1*B*). The differences in MDL between samples from control subjects and patients at the thigh (4.4 ± 0.6 vs. 2.6 ± 0.1 μm, respectively), the proximal leg (3.7 ± 0.3 vs. 2.5 ± 0.2, respectively), and the distal leg (3.6 ± 0.4 vs. 1.9 ± 0.2, respectively) were significantly different, but not in skin from the forearm (3.2 ± 0.3 vs. 2.8 ± 0.1, respectively). There was

a direct, positive correlation between IENF and MDL in patients only at the distal leg. In control subjects there was no significant correlation between IENF and MDL.

Of particular interest was the dichotomy between IENF and MDL in the distal leg and the effect of duration of diabetes. IENF was significantly greater in patients with <5 years' duration of diabetes (37.4 ± 7.1 fibers/mm tissue) than in those with >5 years' duration (7.8 ± 7.1, $P < 0.01$) and was not different from control subjects (34.0 ± 6.0). It should be noted that five of six patients >5 years after diagnosis of diabetes had IENF values of 0. In contrast to IENF, MDL was reduced in a linear fashion in the distal leg from the time of diagnosis. When IENF at the distal leg site was compared with clinical measures of neuropathy taken at the same site, a series of negative correlations were found. Warm ($r = -0.42$, $P < 0.005$) and cold ($r = -0.31$, $P < 0.05$) thermal sense and heat pain thresholds ($r = -0.54$, $P < 0.0001$) all correlated negatively with IENF density. In contrast, there was no correlation between pressure sense and IENF. These results are similar to those found with MDL, in which tests of warm sense ($r = -0.31$, $P < 0.05$), cold sense ($r = -0.36$, $P < 0.02$), and heat pain threshold ($r = -0.46$, $P < 0.002$) were significantly related and pressure sense not significantly related. Finally, there was a significant negative correlation between distal leg IENF, but not MDL, and neuropathy disability scores for total neuropathy ($P < 0.03$) and total sensory ($P < 0.03$) evaluation.

CONCLUSIONS — These data demonstrated both a shortening of IENFs from proximal to distal sites and a significant loss of fibers at the distal site of the lower limb in patients with neuropathy. There were significant changes in IENF in diabetic neuropathy related to duration of diabetes, with a significant fiber loss in patients who were >5 years from diagnosis. In contrast, MDL showed a linear relationship with time since diagnosis of diabetes and with shortening of nerve fibers, irrespective of duration. Furthermore, MDL also showed a shortening of nerve fibers at all sites compared with control subjects. Aging reduced IENF as well as MDL. Finally, in our data there was a correlation between IENF and MDL only at the distal leg and only in neuropathy patients, but not in control subjects, indicating that these patients were at a stage of neuropathy involving only the longest epidermal fibers.

Many studies have demonstrated the loss of peripheral nerve fibers in neuropathy, especially in diabetic patients or even in the pre-diabetic, glucose-intolerant stage. The earliest studies (18,19) used nerve biopsy and both light and electron microscopy techniques to demonstrate the reduction and pathology of distal nerve fibers, especially the sural nerve. Early studies (20,21) with skin biopsy described the pathology of nerve fibers in the lower limb in diabetes, particularly in association with neuropathy. In addition, the shortening of nerve fibers in diabetic neuropathy has previously been reported (12), as well as the correlation of mean fiber length and fiber density. These studies sought to extend the observations to multiple sites and to relate the findings to quantitative sensory tests at the sites of biopsy.

In these patients there was no demonstrable loss of fiber density in the distal upper limb. Quantitative sensory tests performed at the sites of biopsy also showed no deficit in the upper limb, whereas the distal lower limb showed a mild deficit in C-fiber–mediated modalities (warm and cold sense and heat pain) but not in pressure sense, an A-δ fiber–mediated test. Interestingly, the distal fiber loss is seen not just in diabetic neuropathy, but also in a group of patients with other peripheral small-fiber neuropathies. Thus, there is a correlation between gross presentation of neuropathy and the microanatomical evaluation of nerve fibers. This is in concordance with previous reports (4,12,22).

However, our data show that in patients within 5 years of diagnosis there is not significant nerve fiber loss at the distal leg compared with control subjects. This contrasts with reports from other investigators (2,4) that there is a loss of epidermal fibers, even in the pre-diabetic, glucose-intolerant period. These differences may be due to the small numbers of patients analyzed in these studies. Another possibility is that there are separate subgroups of patients who respond in different ways to the onset of metabolic disturbances. Our data in patients <5 years from diagnosis are consistent with a report from Properzi et al. (23) in 1993, who also used skin biopsy, which demonstrated no decrease in PGP 9.5–positive nerve fibers in skin of patients with diabetic neuropathy within 3 years of diagnosis, but an increase in vasoactive intestinal peptide–positive fibers in five of six of the same patients. In addition, Lauria et al. (24) demonstrated a depletion of fibers at the site of deficit, with a normal but disordered nerve fiber distribution in the unaffected area, indicating a more general response to nerve fiber deficits.

The presence of fibers does not address the functional capability of those fibers. The length of the epidermal fibers appears to be linearly related to duration of disease, indicating a slow but steady decline in fiber length in the face of diabetes. This suggests that the mechanisms for reducing fiber length and fiber density are different over time with diabetes, regardless of the overall correlation between the two measures across all groups. MDL may directly reflect the pathological mechanism for reduction of fibers, resulting in shorter and shorter fiber extension, whereas IENF reflects the functional state of epidermal fibers, in which fewer fibers reflects loss of sensation.

One implication of these observations is that they predict difficulty correlating symptom scores and disability scores with epidermal nerve fiber density in the early period after diagnosis. It appears from our data that correlations of IENF with specific quantitative sensory testing measures remain valid. Staining for neurotransmitters such as vasoactive intestinal peptide, calcitonin gene–related peptide, or substance P in combination with PGP 9.5 may provide more detailed functional information.

It has been suggested (22,25) that the use of nerve fiber quantitation in skin biopsies as an early end point in diabetic neuropathy studies might prove useful. Our results would argue against the utility of skin biopsy and measuring IENF alone as a means for detecting and evaluating neuropathic changes in early diabetes, although MDL alone seems promising. There is not a significant difference in PGP 9.5–positive distal leg nerve fibers between control subjects and diabetic neuropathy patients <5 years from diagnosis. Some combination of fiber density and fiber length with neurotransmitter analysis, or even multisite analysis, might prove to be the most revealing method for staging the progression of neuropathy,

using skin biopsy measures as a possible end point in clinical trials.

We have previously demonstrated that the microvascular effects of diabetes mimic premature aging (17), and this may be further confirmation that diabetes complications arise similarly to physiological changes observed with aging. Early studies (25) with PGP 9.5 staining showed that epidermal fiber density loosely correlated with the number of abnormal neurophysiological tests. However, these investigators (26) were subsequently unable to detect consistent, specific neurophysiological deficits related to epidermal fiber loss. In our studies, IENF and MDL correlated with clinical measures of small-fiber neuropathy that reflect small-fiber dysfunction, i.e., thermal perception, but not with clinical tests that reflect large-fiber functions. This is in contrast to a previous report (4) that IENF did not correlate with clinical measures of neuropathy. This may have been due to the smaller number of patients in this study, to the different methodology for evaluating IENF utilized by the authors, or to failure to distinguish effectively between the different nerve fiber types. It appears critical to understand the time course for development of diabetic neuropathy in order to evaluate skin biopsy results. A mix of early and late disease patients might statistically mask the relationship with measurable nerve deficits. If a similar evaluation for the time course of development of nondiabetic neuropathies could be developed, it might be that those patients too would show an initial neurotrophic response in an attempt to overcome developing deficits. This possibility remains to be studied.

It is also important to note that there is a difference in evaluation of IENF dependent on whether immunohistochemistry or immunofluorescence is used. The normal range for IENF in the distal leg in reports from various groups utilizing immunohistochemistry is between 7 and 20 fibers/mm tissue (4,11,14), whereas those groups using confocal microscopy, including our data, report IENF ranges from ~25 to 40 fibers/mm tissue (12,13). Whether this reflects the higher resolution of confocal microscopy, the greater sensitivity of photomultiplier tubes compared to the human eye, or some other methodological issue remains to be resolved.

In summary, we found that IENF and MDL reflect the proximal to distal loss of epidermal nerve fibers described previously by nerve biopsy. These fiber changes correlate negatively with results of quantitative sensory testing, suggesting a direct relationship. However, there was an increase in PGP 9.5–positive IENFs early after the diagnosis of diabetes that was subsequently reduced to nearly zero, whereas the relationship of fiber length to time since diagnosis was linear. In addition, there have been recent reports (9,27) indicating that PGP 9.5 may not stain all epidermal nerve fibers, particularly in neuropathic conditions. Thus, it may be that it will be important to not only measure characteristics of PGP 9.5–positive fibers but also to measure other sensory neurotransmitters in the skin in order to make skin biopsy a useful technique for the early detection of or the efficacy of treatment for diabetic neuropathy.

Acknowledgments— This work was supported by the Diabetes Institutes Foundation, Norfolk, Virginia.

References

1. Vinik AI, Erbas T, Stansberry KB, Pittenger GL: Small fiber neuropathy and neurovascular disturbances in diabetes mellitus. *Exp Clin Endocrinol Diabetes* 109 (Suppl. 2):S451–S473, 2001
2. Sumner CJ, Sheth S, Griffin JW, Cornblath DR, Polydefkis M: The spectrum of neuropathy in diabetes and impaired glucose tolerance. *Neurology* 60:108–111, 2003
3. Vinik AI, Suwanwalaikorn S, Stansberry KB, Holland MT, McNitt PM, Colen LE: Quantitative measurement of cutaneous perception in diabetic neuropathy. *Muscle Nerve* 18:574–584, 1995
4. Smith AG, Ramachandran P, Tripp S, Singleton JR: Epidermal nerve innervation in impaired glucose tolerance and diabetes-associated neuropathy. *Neurology* 57: 1701–***1704, 2001***
5. Thompson RJ, Doran JF, Jackson P, Dhillon AP, Rode J: PGP 9.5: a new marker for vertebrate neurons and neuroendocrine cells. *Brain Res* 278:224–228, 1983
6. Dalsgaard CJ, Rydh M, Haegerstrand A: Cutaneous innervation in man visualized with protein gene product 9.5 (PGP 9.5) antibodies. *Histochemistry* 92:385–390, 1989
7. Polydefkis M, Yiannoutsos CT, Cohen BA, Hollander H, Schifitto G, Clifford DB, Simpson DM, Katzenstein D, Shriver S, Hauer P, Brown A, Haidich AB, Moo L, McArthur JC: Reduced intraepidermal nerve fiber density in HIV-associated sensory neuropathy. *Neurology* 58:115–119, 2002
8. Holland NR, Crawford TO, Hauer P, Cornblath DR, Griffin JW, McArthur JC: Small-fiber sensory neuropathies: clinical course and neuropathology of idiopathic cases. *Ann Neurol* 44:47–59, 1998
9. Petersen KL, Rice FL, Suess F, Berro M, Rowbotham MC: Relief of post-herpetic neuralgia by surgical removal of painful skin. *Pain* 98:119–126, 2002
10. Griffin JW, McArthur JC, Polydefkis M: Assessment of cutaneous innervation by skin biopsies. *Curr Opin Neurol* 14:655–659, 2001
11. McCarthy BG, Hsieh ST, Stocks A, Hauer P, Macko C, Cornblath DR, Griffin JW, McArthur JC: Cutaneous innervation in sensory neuropathies: evaluation by skin biopsy. *Neurology* 45:1848–1855, 1995
12. Kennedy WR, Wendelschafer-Crabb G, Johnson T: Quantitation of epidermal nerves in diabetic neuropathy. *Neurology* 47:1042–1048, 1996
13. Periquet MI, Novak V, Collins MP, Nagaraja HN, Erdem S, Nash SM, Freimer ML, Sahenk Z, Kissel JT, Mendell JR: Painful sensory neuropathy: prospective evaluation using skin biopsy. *Neurology* 53: 1641–1647, 1999
14. Chien HF, Tseng TJ, Lin WM, Yang CC, Chang YC, Chen RC, Hsieh ST: Quantitative pathology of cutaneous nerve terminal degeneration in the human skin. *Acta Neuropathol (Berl)* 102:455–461, 2001
15. Dyck PJ: Detection, characterization and staging of polyneuropathy: assessed in diabetes. *Muscle Nerve* 11:21–32, 1988
16. American Diabetes Association, American Academy of Neurology: Consensus statement: report and recommendations of the San Antonio conference on diabetic neuropathy. *Diabetes Care* 11:592–597, 1988
17. Stansberry KB, Hill MA, Shapiro SA, McNitt PM, Bhatt BA, Vinik AI: Impairment of peripheral blood flow responses in diabetes resembles an enhanced aging effect. *Diabetes Care* 20:1711–1716, 1997
18. Dyck PJ, Lais A, Karnes JL, O'Brien P, Rizza R: Fiber loss is primary and multifocal in sural nerves in diabetic polyneuropathy. *Ann Neurol* 19:425–439, 1986
19. Malik RA, Tesfaye S, Thompson SD, Veves A, Hunter A, Sharma AK, Ward JD, Boulton AJ: Transperineurial capillary abnormalities in the sural nerve of patients with diabetic neuropathy. *Microvasc Res* 48:236–245, 1994
20. Johnson PC, Doll SC: Dermal nerves in human diabetic subjects. *Diabetes* 33: 244–250, 1984
21. Faerman I, Faccio E, Calb I, Razumny J, Franco N, Dominguez A, Podesta HA: Autonomic neuropathy in the skin: a histological study of the sympathetic nerve fibres in diabetic anhidrosis. *Diabetologia*

22:96–99, 1982
22. Polydefkis M, Hauer P, Griffin J, McArthur J: Skin biopsy as a tool to assess distal small fiber innervation in diabetic neuropathy. *Diabetes Technol Ther* 3:23–28, 2001
23. Properzi G, Francavilla S, Poccia G, Aloisi P, Gu XH, Terenghi G, Polak JM: Early increase precedes a depletion of VIP and PGP-9.5 in the skin of insulin-dependent diabetics: correlation between quantitative immunohistochemistry and clinical assessment of peripheral neuropathy. *J Pathol* 169:269–277, 1993
24. Lauria G, Holland N, Hauer P, Cornblath DR, Griffin JW, McArthur JC: Epidermal innervation: changes with aging, topographic location, and in sensory neuropathy. *J Neurol Sci* 164:172–178, 1999
25. Levy DM, Karanth SS, Springall DR, Polak JM: Depletion of cutaneous nerves and neuropeptides in diabetes mellitus: an immunocytochemical study. *Diabetologia* 32:427–433, 1989
26. Levy DM, Terenghi G, Gu X-H, Abraham RR, Springall DR, Polak JM: Immunohistochemical measurements of nerves and neuropeptides in diabetic skin: relationship to tests of neurological function. *Diabetologia* 35:889–897, 1992
27. Pare M, Smith AM, Rice FL: Distribution and terminal arborizations of cutaneous mechanoreceptors in the glabrous finger pads of the monkey. *J Comp Neurol* 445: 347–359, 2002